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D- and L-β-Hydroxybutyrate Dehydrogenases and the Evolution of Ketone Body Metabolism in Gastropod Molluscs

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In vertebrate animals, ketone bodies, synthesized primarily from stored lipid, are important metabolic substrates (1). During starvation, ketone bodies, acetoacetate (Acac) and β-hydroxybutyrate (BHB), are oxidized by some extrahepatic tissues at high rates, and thus perform the important function of sparing limited glycogen stores (1, 2). The enzyme β-hydroxybutyrate dehydrogenase (BHBDH), which catalyzes the interconversion of the ketone bodies, is found in all mammals and most vertebrates, but is absent in most invertebrates (1, 3), including marine molluscs (4). The highest measured BHBDH activities in the animal kingdom, however, are found in the hearts of terrestrial gastropod molluscs (5, 6). We have recently demonstrated that, in tissues of the terrestrial gastropod Cepaea nemoralis, *two unique and previously unknown isoforms of BHBDH occur (5). The isoforms differ from the well-characterized mitochondrial membrane-bound D-BHBDH found in all other animals (7) in that they are cytosolic, and one isoform is specific for the L-enantiomer of BHB. Here we identify patterns in the evolution of these enzyme isoforms in the Gastropoda. BHBDH activities, stereospecificity and subcellular compartmentalization were measured in gastropod species representing four major groups with freshwater and terrestrial representation: Neritomorpha (primitive gilled gastropods), Architaenioglossa (more advanced gilled gastropods), Basommatophora (freshwater pulmonates), and Stylommatophora (terrestrial pulmonates). Mapping of these data onto a phylogeny of the Gastropoda (8) indicates that cytosolic D- and L-BHBDH have arisen a single time, in an ancestral stylommatophoran. All gastropods of the order Stylommatophora possess this unique organization of ketone body metabolism, which has not been found elsewhere in the animal kingdom.*

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Abbreviations: Acac, acetoacetate; BHB, β-hydroxybutyrate, BHBDH, β-hydroxybutyrate dehydrogenase; CCO, cytochrome *C* oxidase; CS, citrate synthase; LDH, lactate dehydrogenase.

Activities and subcellular distributions of D- and L-BHBDH were measured in hepatopancreas and, in some cases, heart and kidney of gastropods by fractionating tissues into mitochondrial and cytosolic fractions, and assaying enzyme activities in each fraction (Table I). Recovery of mitochondria in the "mitochondrial fraction" was 79% or greater for hepatopancreas from all species, based on the distribution of activity of cytochrome *C* oxidase (CCO), an exclusively mitochondrial, membrane-bound enzyme (Table I). A large proportion of these mitochondria maintained structural integrity, as indicated by the low leakage (less than 23% in all cases) of the matrix enzyme citrate synthase (CS). The subcellular distributions of D-BHBDH approximated those of CCO and CS in the hepatopancreas of all Neritopsina, Architaenioglossa, and Basommatophora, with less than 28% of total activity occurring in the cytosolic fraction. In these species, L-BHBDH activity was either not detected or was less than 6% of D-BHBDH activity. The low L-BHBDH activities detected in *Helisoma* and *Physa* were likely the result of contaminating D-BHB, present as an impurity, in the commercial L-BHB preparation (the L-BHB preparation contained up to 0.4% D-BHB). Alternatively, it is possible that these low activities were due to trace levels of L-BHBDH, indicating a transitional state in which both mitochondrial and cytosolic isoforms are present.

The contamination of the mitochondrial fraction of the stylommatophoran *Bradybaena* hepatopancreas with cytosolic lactate dehydrogenase (LDH) was low (7%). The

Table 1

*Activities of β-hydroxybutyrate dehydrogenase (BHBDH) and marker enzymes in mitochondrial and cytosolic compartments of gastropod hepatopancreas**

Genus	Fraction†	Enzyme activity (μmol/min/g wet tissue weight)‡					
		CCO	CS	DL-BHBDH	D-BHBDH	L-BHBDH	LDH
Stylommatophora							
Archachatina							
hepatopancreas	tissue	n.m.	n.m.	n.m.	0.13 ± 0.06	0.77 ± 0.09	n.m.
heart	tissue	n.m.	n.m.	n.m.	35.74 ± 8.60	n.d.	n.m.
kidney	tissue	n.m.	n.m.	n.m.	0.54 ± 0.10	0.58 ± 0.08	n.m.
Bradybaena	mito	1.25 ± 0.10	0.83 ± 0.11	0.14 ± 0.08	0.21 ± 0.12	0.14 ± 0.08	0.33 ± 0.1
	cytosol	0.16 ± 0.06	0.22 ± 0.06	0.85 ± 0.06	0.23 ± 0.08	0.98 ± 0.14	4.35 ± 0.8
Arion	mito	1.10 ± 0.11	1.90 ± 0.10	n.d.	n.d.	n.d.	n.m.
	cytosol	0.03 ± 0.01	0.21 ± 0.04	2.44 ± 0.09	0.07 ± 0.04	2.60 ± 0.11	n.m.
Basommatophora							
Physa	mito	0.87 ± 0.08	4.71 ± 0.50	1.02 ± 0.28	3.07 ± 0.73	n.d.	n.m.
	cytosol	0.17 ± 0.08	1.37 ± 0.34	0.53 ± 0.18	0.99 ± 0.19	0.22 ± 0.04	n.m.
Helisoma	mito	4.85 ± 0.65	3.58 ± 0.29	0.52 ± 0.15	1.94 ± 0.44	n.d.	n.m.
	cytosol	0.04 ± 0.04	0.39 ± 0.08	0.12 ± 0.05	0.13 ± 0.04	0.09 ± 0.01	n.m.
Stagnicola							
hepatopancreas	mito	2.81 ± 0.21	1.40 ± 0.20	2.97 ± 0.14	7.00 ± 0.67	n.d.	0.38 ± 0.2
	cytosol	0.76 ± 0.08	0.18 ± 0.06	0.67 ± 0.10	1.01 ± 0.19	n.d.	1.25 ± 0.2
heart	mito	9.48 ± 0.85	5.72 ± 3.66	n.m.	13.20 ± 1.96	n.d.	6.00 ± 3.3
	cytosol	5.01 ± 2.96	1.95 ± 0.55	n.m.	11.61 ± 1.85	n.d.	34.57 ± 1.7
Neritopsina							
Helicina	mito	6.54 ± 1.24	1.49 ± 0.11	0.45 ± 0.09	0.53 ± 0.32	n.d.	0.17 ± 0.1
	cytosol	0.31 ± 0.11	0.37 ± 0.25	0.25 ± 0.18	0.21 ± 0.10	n.d.	1.72 ± 0.8
Architaenioglossa							
Campeloma	mito	2.12 ± 0.12	1.82 ± 0.11	0.46 ± 0.27	0.65 ± 0.04	n.d.	n.d.
	cytosol	0.01 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.15 ± 0.06	n.d.	n.d.
Pomacea							
hepatopancreas	mito	1.82 ± 0.62	0.89 ± 0.45	1.68 ± 0.63	1.53 ± 0.70	n.d.	0.44 ± 0.2
	cytosol	0.03 ± 0.01	0.25 ± 0.06	0.19 ± 0.11	0.33 ± 0.13	n.d.	0.48 ± 0.01
heart	mito	3.25 ± 1.35	17.05 ± 1.87	n.m.	0.55 ± 0.18	n.d.	0.32 ± 0.1
	cytosol	0.81 ± 0.37	14.18 ± 2.73	n.m.	0.09 ± 0.05	n.d.	7.73 ± 2.3
kidney	mito	2.97 ± 1.83	4.09 ± 0.56	n.m.	0.47 ± 0.09	n.d.	0.44 ± 0.3
	cytosol	0.15 ± 0.10	0.31 ± 0.05	n.m.	0.14 ± 0.10	n.d.	0.92 ± 0.4

* Most gastropods were collected from fields and ponds near the University of Guelph, in Guelph, Ontario, Canada, or purchased (*Pomacea, Campeloma*) from local aquarium stores. The terrestrial prosobranchs, *Helicina orbiculata*, were collected near Jacksonville, Florida. The giant African snails, *Archachatina ventricosa*, were from our laboratory population established with animals provided by the Toronto Metro Zoo. All snails and slugs were kept in terraria or aquaria at room temperature (22° ± 2°C) and fed lettuce. We observed no effect of duration of time spent under these conditions on BHBDH compartmentation or enantiomeric specificity. Taxonomic classification of gastropods was as in Ponder and Lindberg (10) (Table II).

A. ventricosa tissues were not fractionated. They were prepared as in Stuart and Ballantyne. (4) For enzyme measurements in all other gastropods, excised tissues were placed in approximately 20 volumes of sucrose buffer (100 m*M* sucrose, 20 m*M* N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), pH 7.5). Cells were disrupted by five passes of a Potter-Elvejhem homogenizer operated at low speed (<100 rpm). This homogenate was centrifuged at 10,000× *g* for 10 min to separate it into mitochondrial (pellet) and cytosolic (supernatant) fractions. The mitochondrial pellet was resuspended in a volume of buffer equal to the original buffer addition, thus maintaining equal the dilution of both fractions. Both fractions were sonicated with three 5-s bursts of a Vibra-Cell sonicator (Sonics & Materials Inc., Danbury, CT) set to 80% output, 50 watts. These preparations were used directly in the measurement of enzymes.

We used CCO to mark the mitochondrial membrane, CS to mark the leakage of matrix enzymes from mitochondria damaged in the fractionation process, and LDH to evaluate the contamination of the mitochondrial fraction with cytosolic enzymes. CCO, CS, and LDH were measured as in Stuart and Ballantyne. (4) The assays for D-, L- and DL-BHBDH contained 2 m*M* NAD and either 200 m*M* D- or L-BHB or 400 m*M* DL-BHB, respectively (BHB omitted for control) in 50 m*M* imidazole, pH 8.0. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

† "tissue" = whole tissue homogenate; "mito" = mitochondrial fraction; "cytosol" = cytosolic fraction.

‡ CCO = cytochrome *C* oxidase; CS = citrate synthase; LDH = lactate dehydrogenase. DL-, D- and L-BHBDH = activity measured with racemic mixture of DL-β-hydroxybutyrate, high-purity D-β-hydroxybutyrate, and high-purity L-β-hydroxybutyrate, respectively. n.d. = not detected; n.m. = not measured. Values are means ± standard error; $n = 5$.

low L-BHBDH activity observed in the mitochondrial fraction thus can be attributed to cytosolic contamination. Low D-BHBDH activities in *Bradybaena, Arion,* and *Archachatina* hepatopancreas were likely due to contaminating L-BHB in the commercial D-BHB preparation (L-BHB contamination was up to 2%). As we used a [D-BHB] of 20 m*M* in the assays, as much as 0.4 m*M* L-BHB could have been present.

Heart and kidney were fractionated in the same way as the hepatopancreas. Fractionation of heart tissue resulted in a lower recovery of mitochondria and greater leakage into the cytosol of mitochondrial matrix enzymes (Table I), perhaps because of the small size of these tissues (*Stagnicola* hearts averaged 0.8 mg; *Pomacea* hearts averaged 10 mg). Nonetheless, the distribution of BHBDH activity approximated those of CCO and CS in these tissues, indicating that BHBDH activity is localized to the mitochondria in basommatophoran heart and architaenioglossan heart and kidney. In all of these tissues, BHBDH was specific for D-BHB, with no oxidation of L-BHB observed.

The pattern of D- and L-BHBDH distribution in *Archachatina* (Table I) parallels that seen in *Cepaea.* In *C. nemoralis* tissues, no mitochondrial form of BHBDH was found (5). Instead, a cytosolic L-BHBDH was present in hepatopancreas and kidney, and a cytosolic D-BHBDH was found in heart and kidney. Similarly, D-BHB was oxidized by *Archachatina* heart and kidney homogenates and L-BHB was oxidized by kidney and hepatopancreas. This configuration of BHBDH organization appears to be characteristic of the Stylommatophora.

We investigated the evolution of cytosolic BHBDH isoforms by mapping the occurrence of the mitochondrial D-BHBDH and cytosolic L-BHBDH of gastropod hepatopancreas onto a gastropod phylogeny (8) using the MacClade (9) software program (Fig. 1). Mollusc phylogenies based upon morphological (10) and molecular (8, 11–13) data support the monophyly of Gastropoda, within which pulmonates are monophyletic. Within Pulmonata, the Stylommatophora and Basommatophora are each also monophyletic.

For this analysis, we have considered the presence of mitochondrial D-BHBDH to be the ancestral condition. Though BHBDH activity is undetectable in marine gastropods, it is found in all freshwater and terrestrial gastropods that we have studied. Thus, the enzyme appears to have first arisen in a gastropod ancestral to these groups. Our phylogenetic analysis suggests that the cytosolic L-BHBDH isoform evolved a single time in an ancestral stylommatophoran. Gastropods of this order are almost exclusively terrestrial and compose the vast majority of terrestrial snails and slugs (14). To test whether the presence of L-BHBDH correlates with terrestriality, we included the distantly related terrestrial gilled gastropod

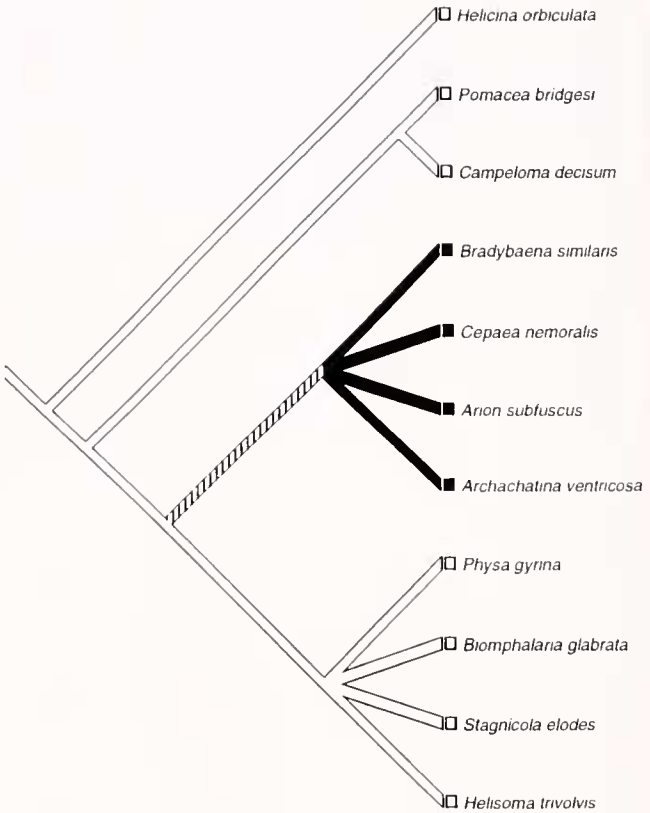

Figure 1. Phylogenetic relationships among freshwater and terrestrial gastropod taxa used in this study. Unfilled lines represent the presence of mitochondrial D-β-hydroxybutyrate dehydrogenase (and absence of L-β-hydroxybutyrate dehydrogenase). Filled lines denote the presence of cytosolic L-β-hydroxybutyrate dehydrogenase (and absence of D-β-hydroxybutyrate dehydrogenase). Hatched line = equivocal occurrence of L-β-hydroxybutyrate dehydrogenase, denoting that the enzyme arose at an undetermined point along this lineage. *Cepaea nemoralis* data are from Stuart and Ballantyne (5). *Biomphalaria glabrata* data are from Meyer *et al.* (15).

Helicina (Neritomorpha) in our analysis. The absence of L-BHBDH in *Helicina,* however, suggests that the occurrence of this isoform correlates exclusively with phylogenetic position.

A general upregulation of BHBDH activities appears to have occurred in tissues of pulmonate gastropods. In pulmonate hearts, exceptionally high activities of D-BHBDH (approximately two orders of magnitude greater than in Pomacea heart) (Table I) suggest that D-BHB is particularly important as a metabolic substrate in these tissues. High activities of all enzymes of ketone body metabolism in *Stagnicola elodes, C. nemoralis,* and *A. ventricosa* indicate a substantial flux through this pathway in all pulmonates. D-BHB levels in hemolymph are as high as those of glucose in the basommatophoran pulmonate *Biomphalaria glabrata* (15). These snails, and tissues isolated from them, actively oxidize ketone bodies. BHB

Table II

Higher classification (10) of gastropod species used for measurements of BHBDH activity

Gastropoda
Orthogastropoda
Neritimorpha
Helicinoidea
Helicina orbiculata (terrestrial)
Apogastropoda
Architaenioglossa
Viviparoidea
Campeloma decisum (freshwater)
Pomacea bridgesi (freshwater)
Heterobranchia
Euthyneura
Pulmonata
Basommatophora
Physa gyrina (freshwater)
Helisoma trivolvis (freshwater)
Stagnicola elodes (freshwater)
Stylommatophora
Archachatina ventricosa (terrestrial)
Bradybaena similaris (terrestrial)
Arion subfuscus (terrestrial)

appears, therefore, to be an important metabolic substrate in pulmonate snails. Unlike mammals, these snails show a decrease in D-BHB levels in the hemolymph during starvation, suggesting that pulmonates may routinely use ketone bodies as energy substrates, whereas mammals confine their use to times of starvation. This difference may be related to a decreased emphasis on amino acid metabolism and a low capacity for extrahepatic oxidation of fatty acids in pulmonates. The metabolic organization of tissues in these organisms suggests that they use ketone bodies which, unlike fatty acids, are freely soluble, as a means of transporting lipid carbon from central stores to peripheral tissues for oxidation.

In stylommatophoran pulmonates, upregulation of BHBDH activity has been followed by the elaboration of new isoforms of the enzyme. This may have occurred through an initial loss of the transmembrane amino acid sequence from the membrane-bound mitochondrial BHBDH, to allow the enzyme to function in the cytosol. In stylommatophoran hepatopancreas and kidney, this enzyme may have been further modified to act upon L-BHB rather than D-BHB. However, this scenario for the occurrence of cytosolic BHBDH isoforms assumes divergence from the ancestral mitochondrial D-BHBDH. Alternatively, the cytosolic enzymes could derive from other proteins, unrelated to the mitochondrial D-BHBDH, and have achieved functional similarity through evolutionary convergence. This convergence appears to have occurred in the evolution of D- and L-LDHs in bacteria (16). On the other hand, divergence of cytosolic L-BHBDH from cytosolic D-BHBDH is suggested by the difficulty of separating these isoforms when they are electrophoresed together on a gel that separates proteins on the bases of size and charge (5). Analyses of primary structures are necessary to determine the relatedness of the three BHBDH isoforms.

The use of both enantiomers of a single metabolic substrate in routine energy metabolism is unusual in the animal kingdom, though other examples of this phenomenon exist among molluscs. Both D- and L-alanine are found in tissues of some marine bivalves. The occurrence of D-alanine appears to be related to a role in osmoregulation (17). Both D- and L-specific isoforms of LDH also occur within individual cephalopods (18), although the physiological significance of these has not been established. The advantages of the stylommatophoran cytosolic BHBDH isoforms are also not immediately obvious. The existence of both D- and L-BHBDH may allow the metabolic partitioning of BHB between specific tissues. Enzyme activities indicate that ketone bodies could be synthesized in the kidney from fatty acids under normal conditions. Both D- and L-BHBDH are present in stylommatophoran kidneys, which are thus able to produce both forms of BHB. Each of these enantiomers of BHB may be specifically targeted to a tissue, with D-BHB being oxidized by heart and L-BHB by hepatopancreas. This adaptation could be related to the apparently greater role for ketone bodies in the intermediary metabolism of pulmonate gastropods—*i.e.*, a refining of a much-used pathway.

The phylogenetic pattern of hepatopancreas BHBDH stereospecificity and subcellular distribution in gastropods suggests that L-BHBDH, and perhaps also D-BHBDH, could be valuable characters for assessing phylogenetic relationships within the Gastropoda. Both enzymes can be rapidly and inexpensively assayed. The presence of hepatopancreas L-BHBDH may be a useful defining characteristic of the Stylommatophora. As such, it will be especially interesting to identify which isoforms of BHBDH are present in tissues of the Archiopulmonata, a group of much-debated phylogenetic position. Certainly, the presence of L-BHBDH in stylommatophoran gastropods should be noted by population geneticists, as staining of electrophoretic gels of gastropod tissues with racemic DL-BHB mixtures will give results that are a function, in part, of the phylogenetic position and tissue of the snail examined.

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